PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 01 February 1999 (01.02.99)	
International application No. PCT/US98/11658	**Applicant's or agent's file reference** 087714/0108
International filing date (day/month/year) 05 June 1998 (05.06.98)	**Priority date** (day/month/year) 06 June 1997 (06.06.97)
Applicant SRIKANTHA, Thyagarajan et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

06 January 1999 (06.01.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Lazar Joseph Panakal

Telephone No.: (41-22) 338.83.38

2452251

Form PCT/IB/331 (July 1992)

19

PATENT COOPERATION TREATY 09/424951

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
087714/0108		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US98/11658	05/06/1998	06/06/1997
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant UNIVERSITY OF IOWA RESEARCH FOUNDATION et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
06/01/1999	14.09.99
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Maucher, C Telephone No. +49 89 2399 7415

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US98/11658

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1,4-14,16-19,21, 22	as originally filed			
2,15	as received on	13/01/1999	with letter of	06/01/1999
3,20	as received on	17/06/1999	with letter of	15/06/1999

Claims, No.:

8	as originally filed			
1-7	as received on	17/06/1999	with letter of	15/06/1999

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-8
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-8
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-8
	No:	Claims	

2. Citations and explanations

see separate sheet

Point V:

The following document (D) is referred to in this communication; the numbering will be adhered to in the rest of the procedure:

D1: JOURNAL OF BACTERIOLOGY,
vol. 178, no. 1, 1996, pages 121-129

D1 discloses a reporter system facilitating the analysis of gene regulation in the developmental programs of C. albicans, one of which being high phenotypic switching (page 121, last sentences of the first paragraph). Therefor, a luciferase gene was fused to the phase specific WH11 (white phase specific) or OP4 (opaque phase specific) promoters of Candida albicans.

1. The subject-matter of claims 1-8 appears to be novel (Article 33(2) PCT), since it is distinguished from the closest prior art (D1) in that "the polynucleotide hybridizes under stringent conditions to the polynucleotide sequence 1" or "3".

2. The technical problem to be solved by the application is how to provide an alternative isolated polynucleotide that codes for a protein that is linked to phenotypic switching in Candida albicans.

   The technical problem is solved by providing a polynucleotide "that hybridizes under stringent conditions to the polynucleotide sequence 1" or "3".

   This solution appears to involve an inventive step (Article 33(3) PCT), since a polynucleotide with such a sequence (CaNik1) could not be derived from any prior art document, either if taken alone or in any combination. Thus, the subject-matter of claims 1-3 appears to meet the requirements of Article 33(3) PCT.

   The same applies to claims 4-8, referring to a method using said polynucleotide.

3. The subject-matter of the documents "MICROBIOLOGY, vol. 144, 1998, pages 425-432" and "JOURNAL OF BACTERIOLOGY, vol. 179, no. 12, 1997, pages

3837-3844" cited in the application refers to relevant subject-matter.

The above documents were published after the present application's priority date, but before its filing date and are therefore relevant for those parts of the present application, if any, which do not have a valid claim to priority.

PATENT COOPERATION TREATY

PCT

09/424951

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT ISA 220) as well as, where applicable, item 5 below.
087714/0108		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 98/11658	05/06/1998	06/06/1997

Applicant

UNIVERSITY OF IOWA RESEARCH FOUNDATION et al.

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1 [ ] **Certain claims were found unsearchable** (see Box I)

2 [ ] **Unity of invention is lacking** (see Box II).

3 [X] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing

- [ ] filed with the international application.
- [X] furnished by the applicant separately from the international application.
  - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
- [ ] Transcribed by this Authority.

4 With regard to the **title**,

- [X] the text is approved as submitted by the applicant.
- [ ] the text has been established by this Authority to read as follows:

5 With regard to the **abstract**,

- [X] the text is approved as submitted by the applicant.
- [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6 The figure of the **drawings** to be published with the abstract is

Figure No.

- [ ] as suggested by the applicant
- [ ] because the applicant failed to suggest a figure
- [ ] because this figure better characterizes the invention

[X] None of the figures

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/11658

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12N9/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SRIKANTHA T ET AL.: "The sea pansy Renilla reniformis luciferase serves as a sensitive bioluminescent reporter for differential gene expression in Candida albicans" JOURNAL OF BACTERIOLOGY, vol. 178, no. 1, 1996, pages 121-129, XP002083236 see the whole document ---	4-8
Y	TIMBERLAKE W E: "CELLULAR REPORTERS FOR ANTIFUNGAL DRUG DISCOVERY" 1995 , PAP. CONFERENCE DISCOVERY MODE ACTION ANTIFUNGAL AGENTS, PAGE(S) 17 - 29 XP000603570 see the whole document ---	4-8

-/--

[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex

Special categories of cited documents

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

& document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 November 1998	27/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. +31-70-340-2040, Tx. 31 651 epo nl, Fax +31-70-340-3016	Knehr, M

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/11658

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 40939 A (CADUS PHARMACEUTICAL CORP ;PAUL JEREMY I (US); BROACH JAMES R (US)) 19 December 1996 see abstract see page 3, line 31 - page 4, line 2 see page 8, line 28 - page 9, line 21 see page 10, line 24 - page 12, line 25 see page 28, line 24 - page 29, line 7 see page 35, line 33 - page 42, line 21; claims 1,12,16-20,24,35-42	4-8
Y	SOLL D R : "Gene regulation during high-frequency switching in Candida albicans" MICROBIOLOGY, vol. 143, 1997, pages 279-288, XP002083237 see the whole document	4-8
A	DATABASE SWISS-PROT Accession number p46588, 15 June 1995 BALL T AND ROSAMOND J: XP002083293 * DNA polymerase III gene (pol3) from Candida albicans * see abstract	1,2
P,X	NAGAHASHI S ET AL.: "Isolation of CaSLN1 and CaNIK1, the genes for osmosensing histidine kinase homologues, from the pathogenic fungus Candida albicans" MICROBIOLOGY, vol. 144, 1998, pages 425-432, XP002083238	1-3
P,Y	see abstract see page 425, column 1, paragraph 1 - page 426, column 2, paragraph 1 see page 430, column 2, paragraph 2; figure 2B	4-8
P,Y	SRIKANTHA T ET AL.: "The WH11 gene of Candida albicans is regulated in two distinct developmental programs through the same transcription activation sequences" JOURNAL OF BACTERIOLOGY, vol. 179, no. 12, 1997, pages 3837-3844, XP002083239 cited in the application see the whole document	4-8

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/11658

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9640939 A	19-12-1996	AU 6110896 A	30-12-1996
		CA 2220459 A	19-12-1996
		EP 0832248 A	01-04-1998

SABE

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

To:
FOLEY & LARDNER
Attn. BENT, Stephen A.
3000 K Street, N.W., Suite 500
Washington, D.C. 20007-5109
UNITED STATES OF AMERICA

ACTION DUE: April 19 Amendments
DOCKETED
RMDR- 1st Mon
U1- 12-07-98
D/I-
DUE 6/27/99
By BN
Date 12/4/98
RETURN TO:

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference 087714/0108	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/US 98/ 11658	International filing date (day/month/year) 05/06/1998
Applicant UNIVERSITY OF IOWA RESEARCH FOUNDATION et al.	

Date of mailing (day/month/year) 27/11/1998

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46).

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Barbara Klaver

Form PCT/ISA/220 (January 1994)

DH 12-14-98

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

(i) the claim is unchanged,

(ii) the claim is cancelled,

(iii) the claim is new,

(iv) the claim replaces one or more claims as filed,

(v) the claim is the result of the division of a claim as filed

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged, claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled, claims 14, 15 and 16 replaced by amended claim 14, claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below
087714/0108		

International application No.	International filing date (day/month/year)	(Earliest) Priority Date (day/month/year)
PCT/US 98/11658	05/06/1998	06/06/1997

Applicant

UNIVERSITY OF IOWA RESEARCH FOUNDATION et al.

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [X] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing

   [ ] filed with the international application.

   [X] furnished by the applicant separately from the international application.

   [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.

   [ ] Transcribed by this Authority

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure

   [ ] because this figure better characterizes the invention.

   [X] None of the figures

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	WO 96 40939 A (CADUS PHARMACEUTICAL CORP ;PAUL JEREMY I (US); BROACH JAMES R (US)) 19 December 1996 see abstract see page 3, line 31 - page 4, line 2 see page 8, line 28 - page 9, line 21 see page 10, line 24 - page 12, line 25 see page 28, line 24 - page 29, line 7 see page 35, line 33 - page 42, line 21; claims 1,12,16-20,24,35-42	4-8
Y	SOLL D R : "Gene regulation during high-frequency switching in Candida albicans" MICROBIOLOGY, vol. 143, 1997, pages 279-288, XP002083237 see the whole document	4-8
A	DATABASE SWISS-PROT Accession number p46588, 15 June 1995 BALL T AND ROSAMOND J: XP002083293 * DNA polymerase III gene (pol3) from Candida albicans * see abstract	1,2
P,X	NAGAHASHI S ET AL.: "Isolation of CaSLN1 and CaNIK1, the genes for osmosensing histidine kinase homologues, from the pathogenic fungus Candida albicans" MICROBIOLOGY, vol. 144, 1998, pages 425-432, XP002083238	1-3
P,Y	see abstract see page 425, column 1, paragraph 1 - page 426, column 2, paragraph 1 see page 430, column 2, paragraph 2; figure 2B	4-8
P,Y	SRIKANTHA T ET AL.: "The WH11 gene of Candida albicans is regulated in two distinct developmental programs through the same transcription activation sequences" JOURNAL OF BACTERIOLOGY, vol. 179, no. 12, 1997, pages 3837-3844, XP002083239 cited in the application see the whole document	4-8

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/11658

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9640939 A	19-12-1996	AU 6110896 A	30-12-1996
		CA 2220459 A	19-12-1996
		EP 0832248 A	01-04-1998

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/11658

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12N9/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	SRIKANTHA T ET AL.: "The sea pansy Renilla reniformis luciferase serves as a sensitive bioluminescent reporter for differential gene expression in Candida albicans" JOURNAL OF BACTERIOLOGY, vol. 178, no. 1, 1996, pages 121-129, XP002083236 see the whole document ---	4-8
Y	TIMBERLAKE W E: "CELLULAR REPORTERS FOR ANTIFUNGAL DRUG DISCOVERY" 1995 , PAP. CONFERENCE DISCOVERY MODE ACTION ANTIFUNGAL AGENTS, PAGE(S) 17 - 29 XP000603570 see the whole document ---	4-8

-/--

[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex

Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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(57) Abstract

a *Candida albicans* gene, *CaNik1*, is involved in phenotypic switching which is significant because of a direct correlation between the switching and the level of virulence of the organism. A method of screening for anti-fungal pharmaceutical candidates entails bringing a test substance into contact with cells containing a *CaNik1* gene or a variant thereof and then monitoring the effect, if any, on the level of expression of the gene.

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CANDIDA ALBICANS TWO-COMPONENT HYBRID KINASE GENE, CaNik1, AND USE THEREOF

BACKGROUND OF THE INVENTION

Candida is an opportunistic yeast that lives in the mouth, throat, intestines, and genitourinary tract of most humans. In a healthy human body, the population of *Candida* is kept in check by the immune system and by a competitive balance with other microorganisms. But when the body's immune system is compromised, as in AIDS patients and in patients undergoing immunosuppressive therapy, *Candida* will grow uncontrolled, leading to systemic infection called "Candida mycosis." If left untreated, such systemic infections frequently lead to the death of the patients.

Candida albicans is a species of particular interest to scientists and doctors because 90% of all cases of Candida mycosis are caused by this species.

At present, the therapy principally available for invasive infections is based on relatively few antimycotics, such as amphotericin B and flucytosine, or the azole derivatives fluconazole and itraconazole. These antimycotics cause serious side effects, such as renal insufficiency, hypocalcaemia and anaemia, as well as unpleasant constitutional symptoms such as fever, shivering and low blood pressure. Amphotericin B is toxic to the kidneys, for example, and yet the pharmaceutical is therapeutic only if administered at dose levels near to being toxic. A discussion of the pharmaceuticals used for treatment and their corresponding side effects can be found, for example, in Boyd, *et al.*, BASIC MEDICAL MICROBIOLOGY (2d ed.), Little, Brown and Company, (1981).

Given the deficiencies of conventional therapies against *Candida*, a need exists for developing pharmaceuticals that are effective in this regard and also safe to use. One step in the development of such pharmaceuticals requires a method for screening compounds in order to identify pharmaceutical candidates.

SUMMARY OF THE INVENTION

It therefore is an object of the present invention to provide an isolated polynucleotide sequence coding for a protein that is linked to phenotypic switching in *Candida albicans*.

It is a further object of the invention to provide a method for screening compounds to identify pharmaceutical candidates for effectively inhibiting the pathogenicity of *C. albicans*.

In accomplishing these and other objects, there has been provided, according to one aspect of the present invention, an isolated polynucleotide that codes for such a protein and that hybridizes, under stringent conditions, to the polynucleotide sequence of SEQ ID No. 1, shown below in Figure 1. In a preferred embodiment, the polynucleotide has the sequence of SEQ ID No. 2 (Figure 2). In another preferred embodiment, the protein displays a kinase activity.

In accordance with another aspect of the present invention, a method is provided for screening compounds to identify pharmaceutical candidates. The inventive method comprises the steps of (A) providing a plurality of cells from yeast species that exhibit phenotypic switching, at least some of which contain (i) a polynucleotide coding for a CaNIK1 protein and (ii) a promoter that is operably linked to the polynucleotide, such that the plurality of cells produces the protein; then (B) bringing the plurality into contact with a test substance; and (C) assessing what effect, if any, the test substance has on the expression of the DNA segment. Assessment step (C) can comprise, for example, of monitoring the level either of the protein or the corresponding mRNA transcript produced by the plurality of cells. In another embodiment, step (C) comprises monitoring the level of kinase activity, within the plurality, that typifies the protein.

In yet another embodiment of the present invention, a promoter is operably linked to a reporter gene. In this context, step (C) comprises monitoring the level of transcription of the reporter gene, after contact between the plurality of cells and the test substance.

Other objects, features and advantages of the present invention will become apparent from the following detailed

description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows the nucleotide sequence (top row) of the PCR product encoding the region spanning the H1 and D domains and the deduced amino acid sequence of the CaNIK1 protein (bottom row). The amino acid residues of functional domains are underlined. The three degenerate primers used to isolate the PCR products are shown as Slb1, Slb2, and Slb3.

FIGURE 2 shows the nucleotide sequence (top row) of the gene *CaNik1* and the deduced primary amino acid sequence of the CaNIK1 protein (bottom row). The beginning of each unique repeat is represented within the rectangle. The potential amino acid residues of different functional domains are underlined.

FIGURE 3 is a schematic representation of the anatomy of two alleles in two strains of *C. albicans* according to the present invention. All the functional domains are shown as white bold letters inside each rectangle. A few of the unique restriction enzyme sites are shown at the top of the rectangle. The start of the protein coding region is shown as ATG. WO-1 and CAI8 are the two strains analyzed in this invention. H1 and H2 are two identical alleles of the strain WO-1. H1-L and H2-S represent large and small alleles respectively in strain CAI8. The five hatched rectangular units in each allele represent repeat units described in this invention. The gray rectangular area encompassing *Xho*I-*Pst*I in H2-S represents the region containing a deletion of approximately one repeat unit length.

FIGURE 4 illustrates the deletion strategy used to generate a homozygous deletion mutant, HH80, in strain CAI8. The region spanning *Afl*II-*Xho*I was deleted and substituted by a hisG-Urablaster cassette in the plasmid pUNIK12.1 to create pCNH35

(Fig. 4c). Plasmid pUNIK12.1 (Fig. 4b) was derived by subcloning a PCR fragment using a pair of primers S1b8 and S1b7R and subcloning into pGEM-T easy plasmid vector. λSA15.1 represent the lambda clone identified in a screen that contain the genomic fragment encompassing the entire *CaNik1* gene and the flanking DNA sequence.

FIGURE 5 shows the deletion strategy used to generate the homozygous deletion mutant in Red 3/6, an ade2⁻ derivative of strain WO-1. The deletion cassette pABX12 (Fig. 5b) was generated by deletion of all the functional domains except H2 and substitution with the *ADE2* gene as an auxotrophic marker in pUNIK12.1 (Fig. 5c). Figure 4 provides a description of λSA15.1.

Table 1 summarizes the effects of the *CaNik1* deletion in HH80 on growth in a variety of solution and conditions, high frequency phenotypic switching, and dimorphism.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Candida albicans is capable of differentiating in a reversible fashion between a bud and a hyphal growth form. Each strain of *C. albicans* can also undergo high frequency phenotypic switching between a limited number of general phenotypes that differ in a variety of traits including putative virulence factors. The frequencies of both of these developmental programs are influenced by environmental conditions. For example, pH and temperature influence the transition between bud and hypha while temperature, UV, white blood cell metabolites and colony aging affect the frequency of high frequency phenotypic switching. The morphological changes made by *C. albicans* in response to environmental cues indicates that the organism uses a sensory mechanism to register and assess environmental alterations.

Autophosphorylating histidine kinases, also known as "two-component response regulators," have been found, in lower eukayotes such as fungi and slime molds, to play a pivotal role in relaying various environmental signals into the cell for inducing appropriate responses and in providing these organisms

with the capacity to respond rapidly to an environmental perturbation. Two-component signal transducers all contain a sensory kinase, which autophosphorylates a histidine residue in response to an environmental cue, and a response regulator, which then is phosphorylated and, through a resultant conformational change, effects a signal that is transduced either directly to a molecular complex, as in the case of the bacterial *CheY* and the flagellar motor, or down a signal transduction pathway, as in the case of *SLN1*. These proteins have been shown to be involved in regulating morphogenesis and development in various prokaryotes and eukaryotes.

That two-component response regulators have been identified in other yeast species suggests that the two-component response regulators may also play a role in the developmental programs of *C. albicans*. The present invention relates to such a two-component response regulator, the hybrid kinase CaNIK1 from *Candida albicans*. A link between the gene encoding *CaNik1* and the processes of phenotypic switching that includes the differential expression of pathogenic genes is evidenced by work with a *CaNik1*-deletion strain of *C. albicans*. See examples 3 and 5. Thus, *CaNik1* is know to be involved in phenotypic switching.

Phenotypic switching is thought to be linked to the virulent characteristics of yeast. *Candida albicans* switches phenotypes with regard to its environment in order to maximize pathogenesis according to the demands of the particular environment. For example, in the WO-1 strain of *Candida albicans*, studies have shown that the yeast is more virulent in its opaque phenotype when located on the skin. When WO-1 is in the white phenotype, however, it is more pathogenic in systemic infections. A description of the relationship between the phenotypic switching and the pathogenic characteristics of *Candida albicans* can be found in Soll, "Switching and Gene Regulation in *Candida albicans*," in SOCIETY FOR GENERAL MICROBIOLOGY SYMPOSIUM 50 (1992). This relationship between phenotypic switching and pathogenicity can be exploited effectively, in a bioassay, for the purpose of discovering pharmaceutical candidates against *Candida albicans*.

1. Definitions

In this description, "**isolated DNA**" is a fragment of DNA that is not integrated in the genomic DNA of an organism. For example, the *CaNik1* gene is a DNA fragment that has been isolated from the genomic DNA of *C. albicans*.

As used herein, "**protein**" refers to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The essential nature of such analogues of naturally occurring amino acids is that, when incorporated into a protein, that protein is specifically reactive to antibodies elicited to the same protein but consisting entirely of naturally occurring amino acids. The terms "polypeptide", "peptide" and "protein" are also inclusive of modifications including, but not limited to, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation. Exemplary modifications are described in most basic texts, such as PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES (2d ed.), T. E. Creighton, W. H. Freeman and Company, New York (1993).

As used herein, "**selectively hybridizes**" includes reference to hybridization, under stringent hybridization conditions, of a nucleic acid sequence to a specified nucleic acid target sequence to a detectably greater degree (e.g., at least 2-fold over background) than its hybridization to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences typically have about at least 80% sequence identity, preferably 90% sequence identity, and most preferably 100% sequence identity (i.e., complementary) with each other.

The terms "**stringent conditions**" or stringent hybridization conditions includes reference to conditions under which a probe will hybridize to its target sequence, to a detectably greater degree than other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions,

target sequences can be identified which are 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth & Wahl, *Anal. Biochem.* 138: 267-84 (1984): T_m = 81.5°C + 16.6 (log M) + 0.41 (%GC) - 0.61 (% form) - 500/L; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq$90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. But severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20 °C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired

degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY --HYBRIDIZATION WITH NUCLEIC ACID PROBES, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays," Elsevier, New York (1993); and in Chapter 2 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing and Wiley-Interscience, New York (1995) (hereafter "Ausubel *et al.*").

Two nucleic acid molecules are considered to have a **"substantial sequence similarity"** if their nucleotide sequences share a similarity of at least 50%. Sequence similarity determinations can be performed, for example, using the FASTA program (Genetics Computer Group; Madison, WI). Alternatively, sequence similarity determinations can be performed using BLASTP (Basic Local Alignment Search Tool) of the Experimental GENIFO(R) BLAST Network Service. See Altschul *et al.*, "Sequence Similarity Searches, Multiple Sequence Alignments, and Molecular Tree Building," in METHODS IN PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY, Glick *et al.* (eds.), pages 251-267 (CRC Press, 1993).

As used herein "**promoter**" includes reference to a region of DNA upstream from the start of transcription and involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. Tissue-specific, tissue-preferred, cell type-specific, and inducible promoters constitute the class of "non-constitutive" promoters. A "constitutive" promoter is one that is active under most environmental conditions.

As used herein "**operably linked**" includes reference to a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary

to join two protein coding regions, contiguous and in the same reading frame.

As used herein, "**expression**" refers to the biosynthesis of a gene product. For example, in the case of a structural gene, expression involves transcription of the structural gene into mRNA and the translation of mRNA into one or more polypeptides.

As used herein, "**expression vector**" is a polynucleotide molecule comprising a gene that is expressed in a host cell. Typically, gene expression is placed under the control of certain regulatory elements, including constitutive or inducible promoters, tissue-specific regulatory elements, and enhancers. Such a gene is said to be "operably linked" to the regulatory elements.

2. Isolating a *Candida albicans* Polynucleotide Segment Encoding *CaNik1* Protein

An endogenous polynucleotide sequence from *Candida albicans* which encodes for the CaNIK1 protein was isolated using a polynucleotide probe derived from PCR amplification. See Example 1. Hybridization of the probe against a genomic library resulted in the determination of the full length polynucleotide sequence encoding the CaNIK1 protein. See Example 2. The full polynucleotide sequence encapsulating the *CaNik1* gene is provided in Figure 2.

3. Nucleic Acids

The present invention provides, *inter alia*, isolated nucleic acids of RNA, DNA, and analogs and/or chimeras thereof, comprising a polynucleotide encoding a CaNIK1 protein or a polynucleotide probe which hybridizes to a polynucleotide encoding CaNIK1 protein. In this regard, the invention provides the nucleotide sequences of Figures 1 and 2. In addition, the present invention also provides other sequences as described below.

a. Polynucleotides Encoding A CaNIK1 Polypeptide or Conservatively Modified or Polymorphic Variants Thereof

As indicated above, the present invention provides isolated heterologous nucleic acids comprising a polynucleotide, wherein the polynucleotide encodes a CaNIK1 protein, disclosed herein in Figure 2, or conservatively modified or polymorphic variants thereof. Those of skill in the art will recognize that the degeneracy of the genetic code allows for a plurality of polynucleotides to encode for the identical amino acid sequence. Such "silent variations" can be used, for example, to selectively hybridize and detect allelic variants of polynucleotides of the present invention. Accordingly, the present invention includes polynucleotides that are silent variations of the polynucleotides of Figure 2. The present invention further provides isolated nucleic acids comprising polynucleotides encoding conservatively modified variants of CaNIK1 encoded by the sequences in Figure 2. Conservatively modified variants can be used to generate or select antibodies immunoreactive to the non-variant polypeptide. Additionally, the present invention further provides isolated nucleic acids comprising polynucleotides encoding one or more polymorphic (allelic) variants of polypeptides/polynucleotides.

b. Polynucleotides That Selectively Hybridize

The present invention also provides isolated nucleic acids comprising polynucleotides, wherein the polynucleotides selectively hybridize, under selective hybridization conditions, to a polynucleotide as discussed above. In this regard, the present invention encompasses polynucleotides that selectively hybridize, under selective conditions, to a polynucleotide as discussed above, excluding the polynucleotide of Figure 2. Thus, the polynucleotides of this embodiment can be used for isolating, detecting, and/or quantifying nucleic acids comprising the polynucleotides described above. For example, polynucleotides of the present invention can be used to identify, isolate, or amplify partial or full-length clones in a deposited library. In some embodiments, the polynucleotides are genomic or cDNA sequences isolated, or otherwise complementary to, a cDNA from a nucleic acid library. Preferably, the cDNA library comprises at

least 80% full-length sequences, preferably at least 85% or 90% full-length sequences, and more preferably at least 95% full-length sequences. The cDNA libraries can be normalized to increase the representation of rare sequences. Low stringency hybridization conditions are typically, but not exclusively, employed with sequences having a reduced sequence identity relative to complementary sequences. Moderate and high stringency conditions can optionally be employed for sequences of greater identity. Low stringency conditions allow selective hybridization of sequences having about 70% sequence identity and can be employed to identify orthologous or paralogous sequences.

c. Polynucleotides Having at Least 60% Sequence Identity

The present invention further provides isolated nucleic acids comprising polynucleotides, wherein the polynucleotides have a specified identity at the nucleotide level to a polynucleotide as disclosed above. In this regard, the present invention encompasses polynucleotides that have a specified identity to the polynucleotides discussed above, but are not the same as the sequence of Figure 2. The percentage of identity to a reference sequence is at least 60% and, rounded upwards to the nearest integer, can be expressed as an integer selected from the group of integers consisting of from 60 to 99. Thus, for example, the percentage of identity to a reference sequence can be at least 70%, 75%, 80%, 85%, 90%, or 95%.

4. Vectors

According to the present invention, the polynucleotide sequence encoding the CaNIK1 protein may be inserted into any suitable yeast vector with any method known to a person who has skill in the art. The vector will typically be comprised of a polynucleotide encoding the CaNIK1 protein operably linked to any suitable promoter which will direct the transcription of the polynucleotide in the intended host cell. Examples of suitable promoters include EF1α2 which is a constitutive promoter and is characterized in Sundstrom *et al.*, *General Bacteriology*, 172: 2036-2045 (1990), and PCK1 which is an inducible promoter and is characterized in Leuker *et al.*, *Gene*

192: 235-240 (1997). According to the present invention, the promoter is operably linked to the polynucleotide encoding for the CaNIK1 protein and inserted into a yeast transformation vector.

Yeast vectors are grouped into five general classes according to their mode of replication in the yeast: YIp, YRp, YCp, YEp, YLp. Comprehensive laboratory techniques regarding insertion of polynucleotides into yeast vectors can be found in Chapter 13 of Ausubel *et al.*

5. Bioassay

Another aspect of the invention is a bioassay useful for screening pharmaceutical candidates which can inhibit pathogenicity in *Candida albicans*. The bioassay is based on assessing a candidate's ability to inhibit expression or functionality of the *CaNik1* gene or its gene product, which as explained above, is linked to the virulent characteristics of the yeast. A bioassay according to the present invention comprises the following steps: transformation of cells from yeast species that exhibit phenotypic switching with a polynucleotide encoding CaNIK1 protein, and a promoter linked to the polynucleotide segment which can drive protein expression; effecting contact between the yeast cells and a pharmaceutical candidate; and analyzing the effect of the pharmaceutical candidate on inhibition of the expression of the *CaNik1* gene. In one embodiment, *C. albicans* cells harboring a *CaNik1* deletion are transformed with a suitable construct containing a CaNIK1-encoding polynucleotide, and an operably linked promoter.

A. Transformation of Yeast Cells

The present invention contemplates the use of yeast cells with a phenotypic switching pathway similar to that of *Candida albicans*. Srikantha *et al.*, J. Bacteriol. 179: 3837-3844 (1997). Transformation of the cells can be accomplished through any means known to a person with skill in the art. One example of a yeast transformation procedure is the lithium acetate procedure whereby yeast cells are briefly incubated in

buffered lithium acetate and transforming DNA is introduced with carrier DNA. Addition of polyethylene glycol (PEG) and a heat shock trigger DNA uptake. An alternate method of transforming yeast cells is the electroporation procedure whereby concentrated cells are transformed using an exponential electric pulse. Comprehensive laboratory techniques regarding yeast transformation procedures can be found in Chapter 13 of Ausubel *et al.*

B. Contact of a Test Substance with Transformed Cells

According to the present invention, a test substance should make contact with at least some of a plurality of cells transformed with a polypeptide encoding *CaNik1*. Contact includes any exposure of the test substance to any surface of a transformed cell. A preferred method of contact would be incubation of the cells with the test substance.

The test substance includes any compound which may have characteristics inhibitory to the growth or the pathogenicity of *Candida albicans*. An example of a test substance is a pharmaceutical compound with antimycotic properties.

6. Assessing of the Effect of the Test Substance on *CaNik1* Gene Expression

According to the present invention, the effect of the pharmaceutical compound on *CaNik1* expression is analyzed after contact between the pharmaceutical compound and the plurality of transformed cells. *CaNik1* expression can be measured through any means known by a person with skill in the art. Examples of methods which monitor the level of gene expression are: measuring levels of CaNIK1 protein and mRNA produced by the cells; or measuring the kinase activity within the cell; or monitoring the level of transcription of a reporter gene operably linked to a promoter.

An example of monitoring *CaNik1* expression is the measurement of levels of CaNIK1 protein produced by the plurality of cells. This can be measured by performing two-dimensional gel electrophoresis using the techniques of isoelectric-focusing and SDS-polyacrylamide gel electrophoresis

followed by autoradiography of the gel. Comprehensive laboratory techniques regarding two-dimensional gel electrophoresis and autoradiography can be found in Chapter 10 and Appendix 3 of Ausubel *et al.*

Another example of monitoring *CaNik1* expression is to measure the level of mRNA encoded within the cell and produced by the plurality. mRNA levels within the cell can be measured with the following three techniques: Northern Blot, primer extension and ribonuclease protection. The Northern Blot procedure consists of fractioning mRNA with gel electrophoresis, transferring the mRNA fragments from the gel onto a filter and hybridizing the target mRNA molecules used a labeled DNA or RNA probe. The primer extension procedure includes hybridizing an oligonucleotide primer to the 5' end of the target mRNA and extending the primer using reverse transcriptase and unlabeled deoxynucleotides to form a single-stranded DNA complementary to the template RNA. The resultant DNA is analyzed on the sequencing gel. The yield of the primer extension product quantifies the amount of mRNA produced by the cell. The ribonuclease protection assay measures mRNA levels by hybridizing sequence specific RNA probes to sample RNAs. The probe anneals to homologous sequences in the sample RNA. The presence of target RNA is analyzed and quantified by gel electrophoresis. Comprehensive laboratory techniques regarding Northern Blot, primer extension and ribonuclease protection assays can be found in Chapter 4 of Ausubel *et al.*

A third example of monitoring *CaNik1* expression is to monitor the level of kinase activity within the plurality of cells. Kinase activity within the cells can be monitored by labeling ATP with ^{32}P in vitro. The labeled ATP acts as the donor substrate, and the CaNIK1 protein acts as the acceptor substrate. Phosphotransfer is detected as the accumulation of ^{32}P-labeled protein within the cell. The accumulation of protein is measured with polyacrylamide gel electrophoresis and autoradiography. Target kinase activity can be distinguished from background kinase activity with autophosphorylation of the CaNIK1 protein on polyacrylamide gel. Comprehensive laboratory techniques regarding

phosphorylation and measurement of kinase activity can be found in Chapter 18 of Ausubel *et al.*

In a further example, a reporter gene is operably linked to a promoter and the level of transcription of the reporter gene is monitored after contact between the plurality and the test substance. In accordance with the present invention, the promoter region of the *CaNik1* gene is operably linked to the luciferase gene. Gene activity is thus linked to luciferase activity, which can then be measured quantitively, with a luminometer, as a bioluminescent reaction.

The present invention is described further below by reference to the following examples, which are illustrative only.

Example 1. PCR Amplification to Determine a *CaNik1* Probe

The following, deoxyinosine-containing, degenerate primers were designed that encompassed the highly conserved regions of the two component response regulators *LemA* (Hrabak & Willis, *J Bacteriol* 174: 3011-3020 (1992)), *BarA* (Nagasawa *et al.*, *Escherichia coli*. *Mol Microbiol*, 6: 799-807 (1992)) and *SLN1* (Ota & Varshavsky, *Science* 262: 566-569 (1993)), respectively: 1) Slb1: 5-GAATTGAGAACGCCTITIAATGG-3, which corresponds to the histidine-autokinase domain; 2) Slb2: 5-AGICCTAAGCCA GTACCACC-3, which corresponds to the ATP-binding domain; and 3) Slb3: 5-TTTAGGCATCTGGACITCCAT, which corresponds to the response regulator domain. Slb1 served as a 5'-end primer for PCR amplifications. The Slb1/Slb2 and Slb1/Slb3 pairs were used to amplify PCR products using the Hot-start wax gem (Perkin, Elmer) protocol. The Hot-start wax gem protocol which generates PCR products used the following reaction mixture: 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.2 mM $MgCl_2$, 100 μM dNTP, 50 μM of each primer and 2.5 units of Taq polymerase, in a final volume of 100 μL. Conditions for PCR cycling included denaturation at 94°C for 1 min, annealing at 40°C for 1.5 min and extension at 72°C for 2.5 min. For all amplifications, *S. cerevisiae* genomic DNA was used as a control for the amplification of the two component hybrid kinase gene *SLN1*, to monitor the quality of the PCR products. PCR products were gel

purified and cloned into either PCR-Trap (Hunter Gen) or pGEM T-Easy (Promega Corp.). Three positive clones were chosen for each of the PCR products of the two sets of primer pairs. pCN.5/3, pCN.5/11 and pCN.5/21 were chosen from the products of S1b1/S1b2; and pCN1.3/5, pCN1.3/13 and pCN 1.3/16 were chosen from the products of S1b1/S1b3.

Example 2. Isolation of *CaNik1* Gene

To isolate a full-length gene, approximately $8x10^4$ plaques of a *C. albicans* genomic library were screened using a 1.2 kb DNA fragment isolated from pCN1.3/13, which spanned the histidine-autokinase (H1) and aspartyl receiver domain (D1). Lambda DNA from 20 positive clones was extracted and Southern blots probed with pCN1.3/13. Using combinations of primer pairs for the arms of the lambda DNA and either the degenerate primers for the histidine-autokinase domain (S1b1) or the response regulator domain (S1b3), lambda clones containing inserts larger than 4 kb were identified. The screen was performed with a high fidelity long PCR protocol (Boehringer Mannheim, Inc., Indianapolis, IN). Three lambda clones contained DNA fragments larger than 3 kb that flanked the upstream region of the histidine-autokinase domain and the downstream region of the aspartyl receiver domain. One of these clones, SA15.1, was chosen to determine the complete nucleotide sequence of the gene in both directions using the ABI automated sequencing system and fluorescent dideoxynucleotides as described earlier.

The DNA fragment generated by S1b1/S1b3 was used as a probe to screen a *C. albicans* EMBL3a lambda genomic library to identify clones containing the full-length gene. Of 10^5 pfu's, twenty positive clones were identified. Clone λSA115.1, which contained a genomic fragment of approximately 4.8 kb with DNA flanking both the H1 and the D domains, was chosen for further characterization. The nucleotide sequence of the DNA insert was determined in both directions. The deduced amino acid sequence revealed an uninterrupted open reading frame of 1081 amino acids beginning with ATG as the initiation codon. The initiation codon was surrounded by an atypical Kozak consensus sequence CTCCAATGA, with cytosine at the -3 position (Kozak,

Nucleic Acids Res, 12: 857-871 (1984)). When total genomic DNA of *C. albicans* strain WO-1 was digested with a variety of restriction enzymes, and the resulting Southern blot hybridized under conditions of high stringency (65°C in Church-Gilbert hybridization buffer) (Church & Gilbert, *Proc Natl Acad. Sci USA* 81: 1991-1995 (1984)) with the 1.2 kb probe spanning the 800 bp upstream of the gene, the banding pattern suggested that Ca*NIK1* is encoded by a single copy gene. When total genomic DNA of strain WO-1 and strain 3153A was digested with *Bsa*AI or *Nci*I and hybridized with the 4.2 kb probe, the patterns were identical, but when *Tsp*I-digested DNA of the two strains were probed, the patterns differed, suggesting allelic differences exist between these strains. A comparison of the *CaNik1* sequence published recently by Nagahashi *et al.*, *Candida albicans*. *Microbology*, 144: 425-432 (1998) for strain IFO1060 and the sequence we obtained for strain WO-1 in the present invention differ at seven nucleotide positions in the open reading frame of 3243 bp.

Example 3. Deletion of *CaNik1* in *C. albicans* Strain CAI8

In order to generate a *CaNik1* deletion cassette, a DNA fragment of approximately 2.1 kb containing both the histidine-autokinase and aspartyl response regulator domains was amplified by PCR using as the template λSA15.1 (Fig. 4a), which contained the 545 bp sequence upstream of the histidine-autokinase domain. The PCR fragment was gel-purified and cloned into the pGEM-T easy vector (Promega). The DNA insert was again excised from the recombinant plasmid with *Eco*RI and subcloned into a PUC18 vector (Life Technologies) at the *Eco*RI site. The resultant recombinant plasmid was designated pUNIK12.1 (Fig. 4b). A deletion construct pCNH35 was generated that spanned the histidine-autokinase and ATP binding-domains. To construct pCNH35, pUNIK12.1 plasmid DNA (Fig. 4b) was digested with *Afl*II and *Xho*I, and blunt-end repaired with the Klenow DNA polymerase I. The resultant plasmid DNA fragment was then gel purified and dephosphorylated with shrimp alkaline phosphatase (US Biochemical). A hisG-URA3-hisG cassette of 3.8 kb from pMB9 was then ligated to derive the disruption cassette

(Fig. 4c). To isolate the *CaNik1* disruption cassette from pCNH35, plasmid DNA was digested with *Pst*I and the digested DNA extracted with phenol: chloroform. Approximately 25 μg of the digestion mixture was used to transform strain CAI8, an *ade2*$^-$ *ura3*$^-$ derivative of wild type strain SC5314, by the lithium acetate protocol. Heterozygotes were selected for growth in minimal medium in the absence of uridine. Transformants were initially tested for the heterozygosity of one of the two *CaNik1* alleles by Southern blot hybridization of genomic DNA digested with *Pst*I. Positive heterozygotes were further confirmed by digesting genomic DNA with *Xho*I and by performing Southern blot hybridization. Because the genomic Southerns revealed polymorphism between the two *CaNik1* alleles, two distinct heterozygotes, NNL6 (L stands for large allele) and NNS7 (S stands for small allele) were selected. The heterozygote NNS7 was chosen to generate the knock-out for the second copy of the Ca*NIK1* gene. Prior to the knock-out of the second copy, NNS7 was subjected to the 5-FOA selection protocol to convert it from uridine prototrophy to auxotrophy. Loss of the *URA3* gene was again confirmed by digestion with *Xho*I and Southern blot analysis. In the final step, a single clone, NNS7.1.1, which was heterozygous for the L allele of the *CaNik1* locus and *URA3*$^+$, was subjected to a second round of transformation with pCNH35, and selected for growth on defined minimal medium lacking uridine. Transformants which had lost the second copy of *CaNik1* were selected by Southern blot hybridization. One of the 125 transformants obtained with the pCNH35-based cassette, HH80, contained a homozygous deletion.

Example 4. *CaNik1* Transcription

To test whether transcription of *CaNik1* was regulated by high frequency phenotypic switching, Northern blots of polyA$^+$mRNA of white and opaque phase cell growth cultures of strain WO-1 were probed with the DNA fragment spanning the H1 and ATP binding domains of *CaNik1*. The *CaNik1* transcript was detectable at very low levels in both white phase and opaque phase cells throughout the exponential phase of growth and in stationary phase. The level of *CaNik1* transcript per cell

remained constant throughout white phase cell growth, but increased steadily during opaque phase cell growth, reaching a level per cell roughly twice that of white phase cells at stationary phase (Fig. 5). Hypha-forming cells of both *C. albicans* strain WO-1 and *C. albicans* strain 3153A contained slightly higher levels of polyA$^+$ *CaNik1* transcript than budding cells. The hypha-to-bud ratio of polyA$^+$-containing *CaNik1* transcript in strain WO-1 and strain 3153A was 1.2 and 1.3, respectively.

Example 5. Functional Characterization of the *CaNik1* Null Mutant of Strain CAI8

To test whether the *CaNik1* deletion mutant HH80 underwent switching, we first had to characterize switching in this strain using a low dose ultraviolet irradiation protocol that increases switching frequencies. Cells were treated with ultraviolet irradiation for 0, 5, 10, 20 and 40 sec, and the percent kill as well as the frequency and type of switch variants were assessed on modified Lee's medium. The proportions of CAI8 and HH80 cells killed after 5, 10, 20, and 40 sec were similar. Identical variant phenotypes were stimulated by UV in both CAI8 and the homozygous deletion strain HH80. However, the frequency of variants induced by comparable levels of UV-irradiation was consistently lower in strain HH80, and this was true in a repeat experiment. For instance, 20 sec of UV irradiation resulted in 10.6% and 2.6% variants in CAI8 and HH80 cells, respectively. These results demonstrate that the *CaNik1* gene product modulates phenotypic switching.

Since deletion of the *nik-1*$^+$ gene in *N. crassa* affects the morphology of hyphae, especially at high osmotic strength (Alex *et al.*, *Proc Natl Acad Sci USA*, 93: 3416-3421 (1996), the capability of the *CaNik1*- minus HH80 strain to form hyphae and the morphology of those hyphae were compared to that of the parent strain CAI8 and a URA3$^+$ isogenic strain CAI8U5 at 0, 1.0 and 1.5 M NaCl. Under the regime of pH-regulated dimorphism, CAI8, CAI8U5, and HH80 cells formed buds at pH 4.5 and hyphae at pH 6.7. The kinetics of evagination for the three strains

at low and high pH were similar at the three tested salt concentrations. At 1.5 M NaCl, the proportion of cells that formed evaginations at low and high pH was dramatically reduced in all three strains. The morphology of the hyphae that formed at pH 6.7 at 0, 1.0, and 1.5 M NaCl were comparable in the three strains. However, there was a significant and reproducible lag in hyphal growth at 1.5M NaCl in HH80 after 300 min. These results demonstrate that the *CaNik1* gene product is not essential for hypha formation under the regime of pH regulated dimorphism, but its presence enhances hypha formation at high ionic strength.

Finally, growth of the *CaNik1* deletion mutant HH80 was tested at 25°C and 37°C for differential sensitivity to osmotic strength and a variety of inhibitors. Patches of budding cells of CAI8, CAI8U5 and HH80 were plated on agar containing modified Lee's medium alone or with one of the following ingredients: 1.0 or 1.5M NaCl; 1M sorbitol; 0.8M KCl; 0.5M Mg_2SO_4; 20 or 40 μg per ml calcofluor; 1, 2 or 4 mg per ml caffeine; 10 or 20 mg per ml hygromycin; 0.002 or 0.004 μg per ml echinocandin; and 0.2 or 0.4M polymyxin B. In three independent experiments, no qualitative differences were observed between the growth of the control strains and the mutant strain HH80 for any of the tested conditions.

All publications and patent applications referred to in this specification are indicative of the level of skill of those in the art to which the invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publications or patent applications were specifically and individually indicated to be incorporated by reference in its entirety.

Other objects, features and advantages of the present invention will become apparent from the foregoing detailed description and examples. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given only by way of illustration.

Table 1

Conditions used to test the effect of gene deletion	*Phenotypic effect in HH80
1. Growth kinetics in	
a) Lee's modified broth	Similar to SC5314, CAI8U5, and CAI8
b) YPD broth	Similar to SC5314, CAI8U5, and CAI8[20].
2. Growth on agar plates with Lee's modified medium or YPD broth supplemented with:	
a) None	++++
b) 1M NaCl	++
c) 1.5M NaCl	+
d) 1M KCl	++
e) 1.2M Sorbitol	++++
f) 0.5m $MgSO_4$	++
g) Caffeine (1-4 mg/mL)	v
h) Calcofluor (20-40 µg/mL)	+++[v]
i) Echinocandin (0.002-0.004 µg/mL)	±
j) 2% Trehalose	++++
k) 2% Raffinose	++++
l) 1M Xylitol	++++
m) 10% Glycerol	++++
3. Switching	
a) spontaneous frequency	No effect
b) UV-stimulated frequency	Decreased
c) repertoire of switch phenoype	No effect
4. Hypha-induction under the regime of pH-regulated dimorphism.	
with no osmotic shock:	
a) time for 50% evagination	No effect
b) morphology of hypha	No effect
c) growth of hyphal filaments	
with osmotic shock using 1.5M NaCl	
i) time for 50% evaginations	decreased in both wild type and the mutant
ii) morphology of hyphae	no difference between wild hyphae and the mutant
iii) growth of hyphal filaments	the growth of the hyphae after 300 min was reduced in the mutant as compared to that in wild type

Table 1 (cont'd)

In order to asses the effect of gene deletion on growth, exponentially grown cells of wild type (SC5314), parental auxotrophic strain used to delete *NIK1* gene (CAI8), URA3$^+$ derivative of CAI8 (CAI8U5) and homozygous deletion mutant (HH80) were serially diluted and spot plated on agar plates with or without supplements in the medium. In all the growth medium used in this study, 2% glucose served as a carbon source except in the growth medium containing raffinose, trehalose and glycerol. The symbol "v" denote variable growth. Growth of the cultures were qualitatively assessed as very good (++++), good (+++), fair (++), poor (+), poor to no growth (±). o indicates that colonies were very small (less than 1mm) as assessed by the colony size on agar plates spread with cultures to generate 50 to 100 individual colonies. The growth of the cultures were assessed after 2 or 3 days incubation both at 25°C and 37°C.

WHAT IS CLAIMED IS:

1. An isolated polynucleotide that codes for a protein that is linked to phenotypic switching in *Candida albicans* and that hybridizes, under stringent conditions, to polynucleotide sequence of SEQ ID No. 1.

2. A polynucleotide according to claim 1, having the sequence of SEQ ID No. 2.

3. A polynucleotide according to claim 1, wherein said protein displays kinase activity.

4. A method for screening compounds to identify pharmaceutical candidates, comprising the steps of (A) providing a plurality of cells from yeast species exhibiting phenotypic switching, at least some of which contain (i) a polynucleotide according to claim 1 and (ii) a promoter that is operably linked to said polynucleotide, such that said plurality produces said protein; then (B) bringing said plurality into contact with a test substance; and (C) assessing what effect, if any, said test substance has on the expression of said DNA segment.

5. A method according to claim 4, wherein step (C) comprises monitoring the level of said protein produced by said plurality.

6. A method according to claim 4, wherein step (C) comprises monitoring the level of mRNA encoded by said DNA and produced by said plurality.

7. A method according to claim 4, wherein step (C) comprises monitoring the level of kinase activity within said plurality, wherein said kinase activity typifies said protein.

8. A method according to claim 4, wherein a promoter is operably linked to a reporter gene and wherein step (C) comprises monitoring the level of transcription of the reporter gene after contact between said plurality and said test substance.

```
1531      Slb1------>
GAGATTAGAACACCATTGAATGGGATTATTGGWATGACYCAGTTGTCRCTTGATACAGAG  1590
GluIleArgThrProLeuAsnGlyIleIleGlyMetThrGlnLeuSerLeuAspThrGlu   530
                  H1
TTGACRCAGTACCAACGAGAGATGTTGTCGATTGTGCATAACTTGGCAAATTCCTTGTTG  1650
LeuThrGlnTyrGlnArgGluMetLeuSerIleValHisAsnLeuAlaAsnSerLeuLeu   550

ACCATTATAGACGATATATTGGATATTTCTAAGATTGAGGCGAATAGAATGACGGTGGAA  1710
ThrIleIleAspAspIleLeuAspIleSerLysIleGluAlaAsnArgMetThrValGlu   570

CAGATTGATTTTTCATTAAGAGGGACAGTGTTTGGTGCATTGAAAACGTTAGCCGTCAAA  1770
GlnIleAspPheSerLeuArgGlyThrValPheGlyAlaLeuLysThrLeuAlaValLys   590

GCTATTGAAAAAAACCTAGACTTGACCTATCAATGTGATTCATCGTTTCCAGATAATCTT  1830
AlaIleGluLysAsnLeuAspLeuThrTyrGlnCysAspSerSerPheProAspAsnLeu   610

ATTGGAGATAGTTTTAGATTACGACAAGTTATTCTTAACTTGGCTGGTAATGCTATTAAG  1890
IleGlyAspSerPheArgLeuArgGlnValIleLeuAsnLeuAlaGlyAsnAlaIleLys   630
                                    N
TTTACTAAAGAGGGGAAAGTTAGTGTTAGTGTGAAAAAGTCTGATAAAATGGTGTTAGAT  1950
PheThrLysGluGlyLysValSerValSerValLysLysSerAspLysMetValLeuAsp   650

AGTAAGTTGTTGTTAGAGGTTTGTGTTAGCGACACGGGAATAGGTATAGAGAAAGACAAA  2010
SerLysLeuLeuLeuGluValCysValSerAspThrGlyIleGlyIleGluLysAspLys   670
                              G1
TTGGGATTGATTTTCGATACCTTCTGTCAAGCTGATGGTTCTACTACAAGAAAGTTTGGT  2070
LeuGlyLeuIlePheAspThrPheCysGlnAlaAspGlySerThrThrArgLysPheGly   690
  <------Slb2
GGTACAGGTTTAGGGTTGTCAATTTCCAAACAGTTGATACATTTAATGGGTGGAGAGATA  2130
GlyThrGlyLeuGlyLeuSerIleSerLysGlnLeuIleHisLeuMetGlyGlyGluIle   710
G2
TGGGTTACTTCGGAGTATGGATCCGGRTCAAACTTTTATTTTACGGTGTGCGTGTCGCCA  2190
TrpValThrSerGluTyrGlySerGlySerAsnPheTyrPheThrValCysValSerPro   730

TCTAATATTAGATATACTCGACAAACCGAACAATTGTTACCATTTAGTTCCCATTATGTG  2250
SerAsnIleArgTyrThrArgGlnThrGluGlnLeuLeuProPheSerSerHisTyrVal   750

TTATTTGTATCGACTGAGCATACTCAAGAAGAACTTGATGTGTTGAGAGATGGAATTATA  2310
LeuPheValSerThrGluHisThrGlnGluGluLeuAspValLeuArgAspGlyIleIle   770

AAACTTGGATTGATACCTATAATAGTGAGAAATATTGAAGATGCAACATTGACTGAGCCG  2370
GluLeuGlyLeuIleProIleIleValArgAsnIleGluAspAlaThrLeuThrGluPro   790

GTGAAATATGATATAATTATGATTGATTCGATAGAGATTGCCAAAAAGTTGAGGTTGTTA  2430
ValLysTyrAspIleIleMetIleAspSerIleGluIleAlaLysLysLeuArgLeuLeu   810

TCGGAGGTTAAATATATTCCGTTGGTTTTGGTCCATCATTCTATTCCACAGTTGAATATG  2490
SerGluValLysTyrIleProLeuValLeuValHisHisSerIleProGlnLeuAsnMet   830

AGAGTATGTATTGATTTGGGGATATCTTCCTATGCAAATACGCCATGTTCGATCACGGAC  2550
ArgValCysIleAspLeuGlyIleSerSerTyrAlaAsnThrProCysSerIleThrAsp   850

TTGGCCAGTGCGATTATACCAGCGTTGGAGTCGAGATCTATATCACAGAACTCAGACGAG  2610
LeuAlaSerAlaIleIleProAlaLeuGluSerArgSerIleSerGlnAsnSerAspGlu   870

TCGGTGAGGTACAAAATATTACTAGCAGAGGACAACCTCGTCAATCAGAAACTTGCAGTT  2670
SerValArgTyrLysIleLeuLeuAlaGluAspAsnLeuValAsnGlnLysLeuAlaVal   890
```

Fig. 1a

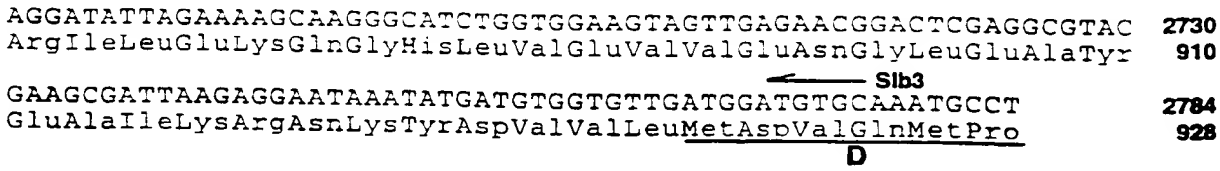
AGGATATTAGAAAAGCAAGGGCATCTGGTGGAAGTAGTTGAGAACGGACTCGAGGCGTAC 2730
ArgIleLeuGluLysGlnGlyHisLeuValGluValValGluAsnGlyLeuGluAlaTyr 910
Slb3
GAAGCGATTAAGAGGAATAAATATGATGTGGTGTTGATGGATGTGCAAATGCCT 2784
GluAlaIleLysArgAsnLysTyrAspValValLeuMetAspValGlnMetPro 928
D

Fig. 1b

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ATGAACCCCACTAAAAAACCTCGGTTATCACCAATGCAGCCCTCTGTTTTTGAAATACTC   60
MetAsnProThrLysLysProArgLeuSerProMetGlnProSerValPheGluIleLeu   20

AACGACCCTGAGCTTTATAGTCAGCACTGTCATAGCCTTAGGGAAACACTTCTTGATCAT  120
AsnAspProGluLeuTyrSerGlnHisCysHisSerLeuArgGluThrLeuLeuAspHis   40

TTCAACCATCAAGCTACACTTATCGACACTTATGAACATGAACTAGAAAAATCCAAAAAT  180
PheAsnHisGlnAlaThrLeuIleAspThrTyrGluHisGluLeuGluLysSerLysAsn   60

GCCAACAAAGCGTCCCAACAAGCACTTAGTGAAATAGGTACAGTTGTTATATCTGTTGCC  240
AlaAsnLysAlaSerGlnGlnAlaLeuSerGluIleGlyThrValValIleSerValAla   80

ATGGGAGACTTGTCGAAAAAAGTTGAGATTCACACAGTAGAAAATGACCCTGAGATTTTA  300
MetGlyAspLeuSerLysLysValGluIleHisThrValGluAsnAspProGluIleLeu  100

AAAGTCAAAATCACCATCAACACCATGATGGATCAATTACAGACATTTGCTAATGAGGTT  360
LysValLysIleThrIleAsnThrMetMetAspGlnLeuGlnThrPheAlaAsnGluVal  120

ACAAAAGTCGCCACCGAAGTCGCAAATGGTGAACTAGGTGGACAAGCGAAAAATGATGGA  420
ThrLysValAlaThrGluValAlaAsnGlyGluLeuGlyGlyGlnAlaLysAsnAspGly  140

TCTGTTGGTATTTGGAGATCACTTACAGACAATGTTAATATTATGGCTCTTAATTTAACT  480
SerValGlyIleTrpArgSerLeuThrAspAsnValAsnIleMetAlaLeuAsnLeuThr  160

AACCAAGTGCGAGAAATTGCTGATGTCACACGTGCTGTTGCCAAGGGGGACTTGTCACGT  540
AsnGlnValArgGluIleAlaAspValThrArgAlaValAlaLysGlyAspLeuSerArg  180

AAAATTAATGTACACGCCCAGGGTGAAATCCTTCAACTTCAACGTACAATAAACACCATG  600
LysIleAsnValHisAlaGlnGlyGluIleLeuGlnLeuGlnArgThrIleAsnThrMet  200

GTGGATCAGTTACGAACGTTTGCATTCGAAGTATCTAAAGTTGCTAGAGATGTTGGTGTG  660
ValAspGlnLeuArgThrPheAlaPheGluValSerLysValAlaArgAspValGlyVal  220

CTTGGTATATTAGGAGGACAAGCGTTGATTGAAAATGTTGAAGGTATTTGGGAAGAGTTG  720
LeuGlyIleLeuGlyGlyGlnAlaLeuIleGluAsnValGluGlyIleTrpGluGluLeu  240

ACTGATAATGTCAATGCCATGGCTCTTAATTTGACTACACAAGTGAGAAATATTGCCAAT  780
ThrAspAsnValAsnAlaMetAlaLeuAsnLeuThrThrGlnValArgAsnIleAlaAsn  260

TCACCACTGCCGTTGCCAAGGGGGATTTGTCGAAAAAAGTCACTGCTGATTGTAAGGGA  840
ValThrThrAlaValAlaLysGlyAspLeuSerLysLysValThrAlaAspCysLysGly  280

GAAATYCTTGATTTGAAACTTACTATTAATCAAATGGTGGACCGATTACAGAATTTTGCT  900
GluIleLeuAspLeuLysLeuThrIleAsnGlnMetValAspArgLeuGlnAsnPheAla  300

CTTGCGGTGACGACATTGTCGAGAGAGGTTGGTACTTTGGGTATTTTGGGTGGACAAGCT  960
LeuAlaValThrThrLeuSerArgGluValGlyThrLeuGlyIleLeuGlyGlyGlnAla  320

AACGTACAGGATGTTGAAGGTGCTTGGAAACAGGTTACAGAAAATGTCAACCTAATGGCT 1020
AsnValGlnAspValGluGlyAlaTrpLysGlnValThrGluAsnValAsnLeuMetAla  340

ACTAATTTAACTAACCAAGTGAGATCTATTGCTACAGTTACTACTGCAGTTGCGCATGGT 1080
ThrAsnLeuThrAsnGlnValArgSerIleAlaThrValThrThrAlaValAlaHisGly  360

GATTTGTCGCAAAAGATTGATGGTCATCCCAAAGGAGAGATTTTACAATTGAAAAATACA 1140
AspLeuSerGlnLysIleAspGlyHisProLysGlyGluIleLeuGlnLeuLysAsnThr  380
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Fig. 2a

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ATCAACAAGATGGTGGACTCTTTGCAGTTGTTTGCATCAGAAGTGTCGAAAGTGGCACAA 1200
IleAsnLysMetValAspSerLeuGlnLeuPheAlaSerGluValSerLysValAlaGln  400

GATGTTGGTATTAATGGAAAATTAGGTATTCAAGCACAAGTTAGTGATGTTGATGGATTA 1260
AspValGlyIleAsnGlyLysLeuGlyIleGlnAlaGlnValSerAspValAspGlyLeu  420

TGGAAGGAGATTACGTCTAATGTAAATACCATGGCTTCAAATTTAACTTCGCAAGTGAGA 1320
TrpLysGluIleThrSerAsnValAsnThrMetAlaSerAsnLeuThrSerGlnValArg  440

GCTTTTGCACAGATTACTGCTGCTGCTACTGATGGGGATTTCACTAGATTTATTACTGTT 1380
AlaPheAlaGlnIleThrAlaAlaAlaThrAspGlyAspPheThrArgPheIleThrVal  460

GAAGCACTGGGAGAGATGGATGCGTTGAAAACAAAGATTAATCAAATGGTGTTTAACTTA 1440
GluAlaLeuGlyGluMetAspAlaLeuLysThrLysIleAsnGlnMetValPheAsnLeu  480

AGGGAATCGCTTCAAAGGAATACTGCGGCTAGAGAAGCTGCTGAGTTGGCCAATAGTGCG 1500
ArgGluSerLeuGlnArgAsnThrAlaAlaArgGluAlaAlaGluLeuAlaAsnSerAla  500

AAATCCGAGTTTTTAGCAAACATGTCGCATGAGATTAGAACACCATTGAATGGGATTATT 1560
LysSerGluPheLeuAlaAsnMetSerHisGluIleArgThrProLeuAsnGlyIleIle  520
                                                    H1

GGWATGACYCAGTTGTCRCTTGATACAGAGTTGACRCAGTACCAACGAGAGATGTTGTCG 1620
GlyMetThrGlnLeuSerLeuAspThrGluLeuThrGlnTyrGlnArgGluMetLeuSer  540

ATTGTGCATAACTTGGCAAATTCCTTGTTGACCATTATAGACGATATATTGGATATTTCT 1680
IleValHisAsnLeuAlaAsnSerLeuLeuThrIleIleAspAspIleLeuAspIleSer  560

AAGATTGAGGCGAATAGAATGACGGTGGAACAGATTGATTTTTCATTAAGAGGGACAGTG 1740
LysIleGluAlaAsnArgMetThrValGluGlnIleAspPheSerLeuArgGlyThrVal  580

TTTGGTGCATTGAAAACGTTAGCCGTCAAAGCTATTGAAAAAAACCTAGACTTGACCTAT 1800
PheGlyAlaLeuLysThrLeuAlaValLysAlaIleGluLysAsnLeuAspLeuThrTyr  600

CAATGTGATTCATCGTTTCCAGATAATCTTATTGGAGATAGTTTTAGATTACGACAAGTT 1860
GlnCysAspSerSerPheProAspAsnLeuIleGlyAspSerPheArgLeuArgGlnVal  620

ATTCTTAACTTGGCTGGTAATGCTATTAAGTTTACTAAAGAGGGGAAAGTTAGTGTTAGT 1920
IleLeuAsnLeuAlaGlyAsnAlaIleLysPheThrLysGluGlyLysValSerValSer  640
          N

GTGAAAAAGTCTGATAAAATGGTGTTAGATAGTAAGTTGTTGTTAGAGGTTTGTGTTAGC 1980
ValLysLysSerAspLysMetValLeuAspSerLysLeuLeuLeuGluValCysValSer  660

GACACGGGAATAGGTATAGAGAAAGACAAATTGGGATTGATTTTCGATACCTTCTGTCAA 2040
AspThrGlyIleGlyIleGluLysAspLysLeuGlyLeuIlePheAspThrPheCysGln  680
G1

GCTGATGGTTCTACTACAAGAAAGTTTGGTGGTACAGGTTTAGGGTTGTCAATTTCCAAA 2100
AlaAspGlySerThrThrArgLysPheGlyGlyThrGlyLeuGlyLeuSerIleSerLys  700
                                    G2

CAGTTGATACATTTAATGGGTGGAGAGATATGGGTTACTTCGGAGTATGGATCCGGRTCA 2160
GlnLeuIleHisLeuMetGlyGlyGluIleTrpValThrSerGluTyrGlySerGlySer  720

AACTTTTATTTTACGGTGTGCGTGTCGCCATCTAATATTAGATATACTCGACAAACCGAA 2220
AsnPheTyrPheThrValCysValSerProSerAsnIleArgTyrThrArgGlnThrGlu  740

CAATTGTTACCATTTAGTTCCCATTATGTGTTATTTGTATCGACTGAGCATACTCAAGAA 2280
GlnLeuLeuProPheSerSerHisTyrValLeuPheValSerThrGluHisThrGlnGlu  760

GAACTTGATGTGTTGAGAGATGGAATTATAGAACTTGGATTGATACCTATAATAGTGAGA 2340
GluLeuAspValLeuArgAspGlyIleIleGluLeuGlyLeuIleProIleIleValArg  780
```

Fig. 2b

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AATATTGAAGATGCAACATTGACTGAGCCGGTGAAATATGATATAATTATGATTGATTCG 2400
AsnIleGluAspAlaThrLeuThrGluProValLysTyrAspIleIleMetIleAspSer  800

ATAGAGATTGCCAAAAAGTTGAGGTTGTTATCGGAGGTTAAATATATTCCGTTGGTTTTG 2460
IleGluIleAlaLysLysLeuArgLeuLeuSerGluValLysTyrIleProLeuValLeu  820

GTCCATCATTCTATTCCACAGTTGAATATGAGAGTATGTATTGATTTGGGGATATCTTCC 2520
ValHisHisSerIleProGlnLeuAsnMetArgValCysIleAspLeuGlyIleSerSer  840

TATGCAAATACGCCATGTTCGATCACGGACTTGGCCAGTGCGATTATACCAGCGTTGGAG 2580
TyrAlaAsnThrProCysSerIleThrAspLeuAlaSerAlaIleIleProAlaLeuGlu  860

TCGAGATCTATATCACAGAACTCAGACGAGTCGGTGAGGTACAAAATATTACTAGCAGAG 2640
SerArgSerIleSerGlnAsnSerAspGluSerValArgTyrLysIleLeuLeuAlaGlu  880

GACAACCTCGTCAATCAGAAACTTGCAGTTAGGATATTAGAAAAGCAAGGGCATCTGGTG 2700
AspAsnLeuValAsnGlnLysLeuAlaValArgIleLeuGluLysGlnGlyHisLeuVal  900

CAAGTAGTTGAGAACGGACTCGAGGCGTACGAAGCGATTAAGAGGAATAAATATGATGTG 2760
GluValValGluAsnGlyLeuGluAlaTyrGluAlaIleLysArgAsnLysTyrAspVal  920

GTGTTGATGGATGTGCAAATGCCTGTAATGGGTGGGTTTGAAGCTACGGAGAAGATTCGA 2820
ValLeuMetAspValGlnMetProValMetGlyGlyPheGluAlaThrGluLysIleArg  940
          D

CAATGGGAGAAAAAGTCTAACCCAATTGACTCGTTGACGTTTAGGACTCCAATTATTGCC 2880
GlnTrpGluLysLysSerAsnProIleAspSerLeuThrPheArgThrProIleIleAla  960

CTCACTGCACACGCCATGTTAGGTGATAGAGAAAAGTCATTGGCCAAGGGGATGGACGAT 2940
LeuThrAlaHisAlaMetLeuGlyAspArgGluLysSerLeuAlaLysGlyMetAspAsp  980

TATGTGAGTAAGCCATTGAAGCCGAAATTGTTAATGCAGACGATAAACAAGTGTATTCAT 3000
TyrValSerLysProLeuLysProLysLeuLeuMetGlnThrIleAsnLysCysIleHis 1000
                                          H2

AATATTAACCAGTTGAAAGAATTGTCGAGAAATAGTAGGGGTAGCGATTTTGCAAAGAAG 3060
AsnIleAsnGlnLeuLysGluLeuSerArgAsnSerArgGlySerAspPheAlaLysLys 1020

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GACATGATTGGGGACACTCCCCGTCAAGGGAGTGTGGAGGGAGGGGGTACAAGTAGTAGA 3180
AspMetIleGlyAspThrProArgGlnGlySerValGluGlyGlyGlyThrSerSerArg 1060

CCAGTACAGAGAAGGTCTGCCAGGGAGGGGTCGATCACTACAATTAGTGAACAAATCGAC 3240
ProValGlnArgArgSerAlaArgGluGlySerIleThrThrIleSerGluGlnIleAsp 1080

CGTTAG                                                         3246
Arg***                                                         1082
```
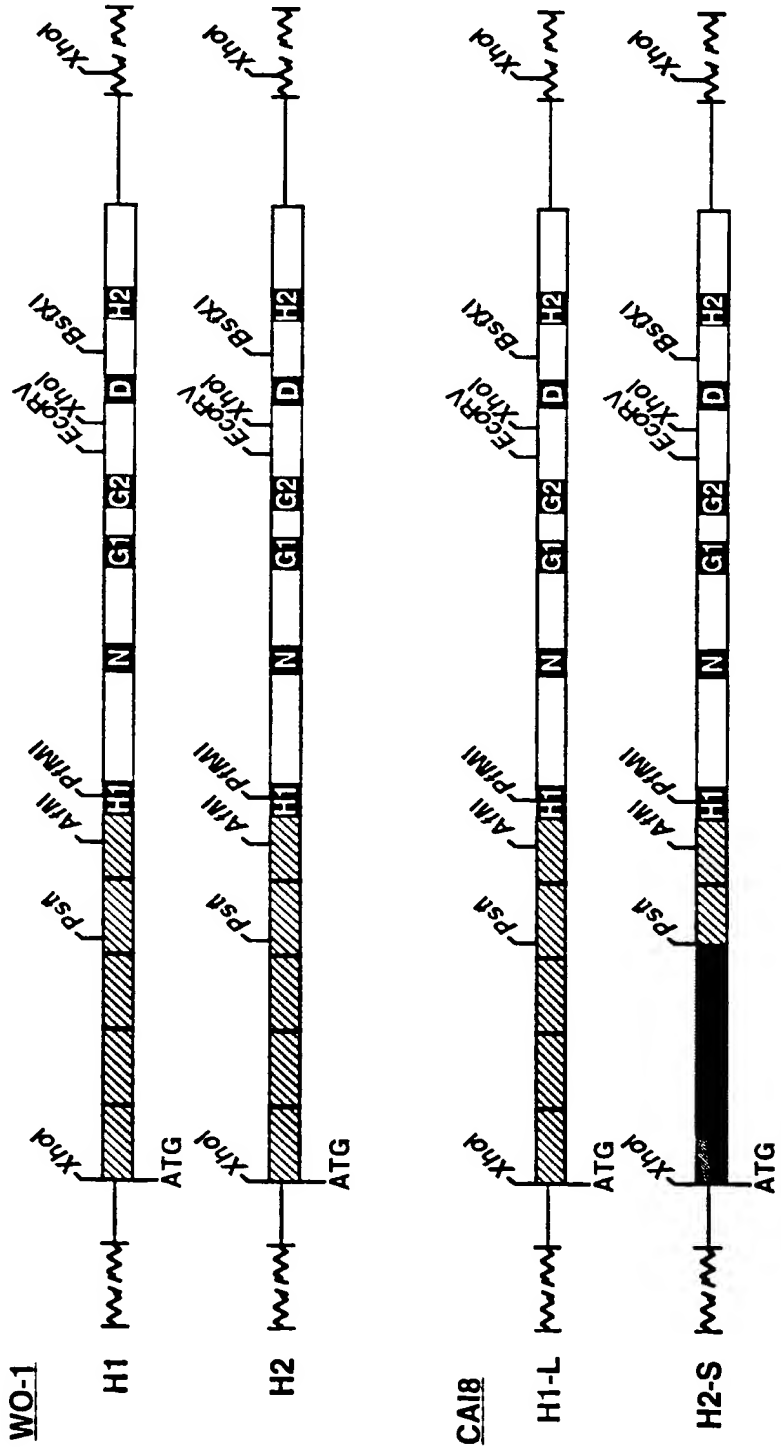

Fig. 2c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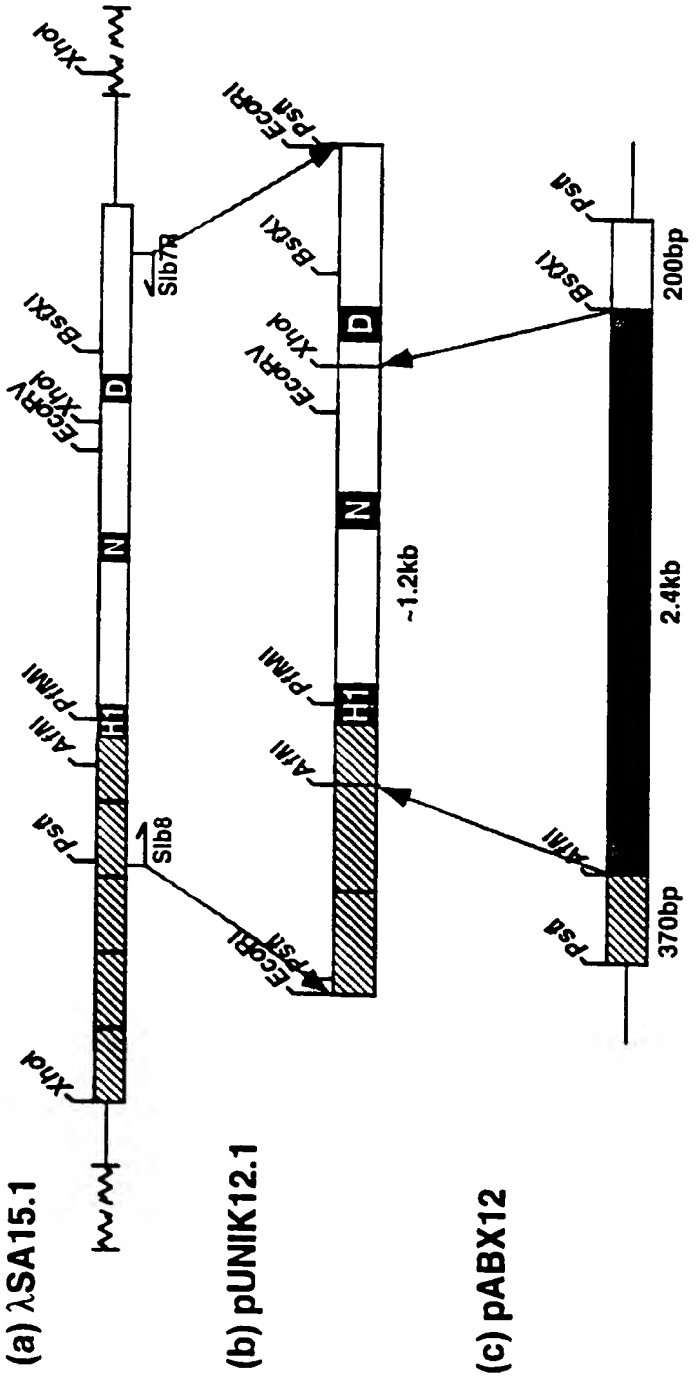
H2
CAI8
H1-L
H2-S
XhoI
BsaXI
EcoRV
XhoI
H2
D
G2
G1
N
H1
PflMI
AflII
PsiI
XhoI
ATG

Fig. 3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(b) pUNIK12.1
(c) pABX12
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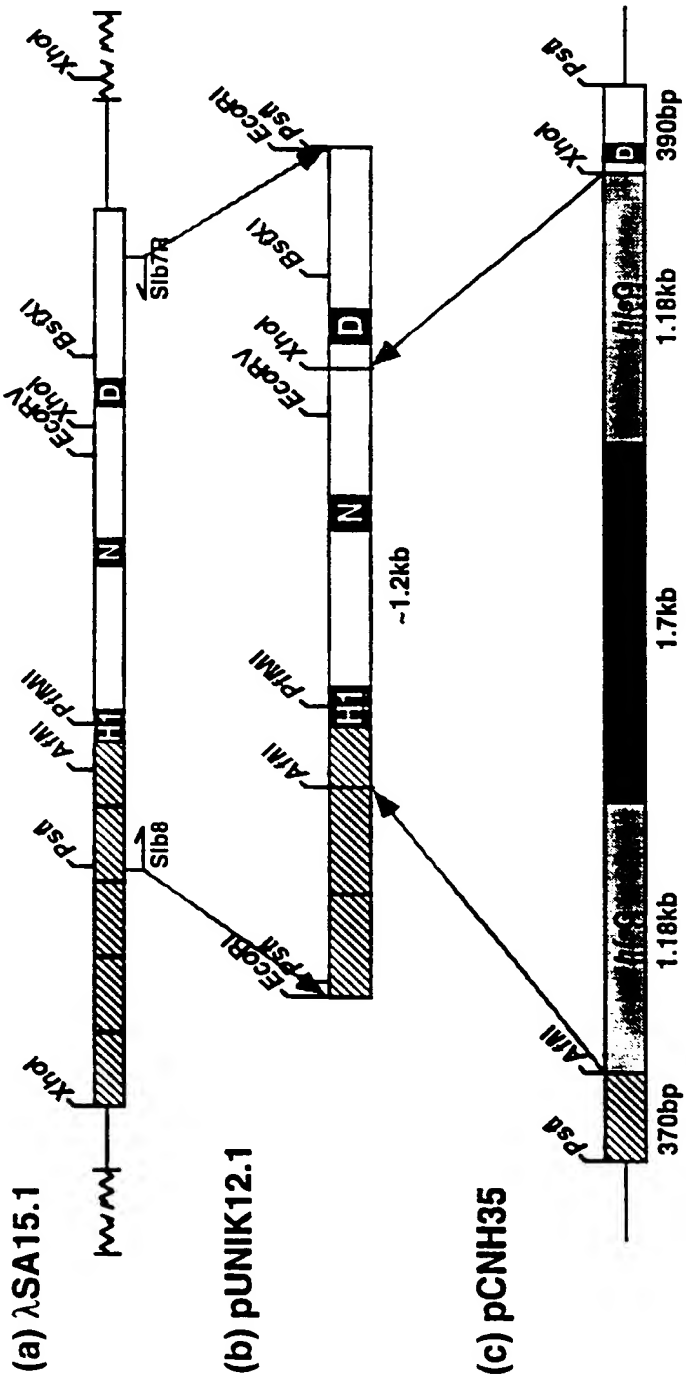
EcoRI
PstI
Slb7
BsaXI
EcoRV
D
N
H1
PflMI
AflII
Slb8
~1.2kb
200bp
2.4kb
370bp

Fig. 4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(b) pUNIK12.1
(c) pCNH35
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EcoRI
BstXI
EcoRV
PflMI
AflI
SlbR
Slb8
D
N
H1
~1.2kb
390bp
1.18kb
1.7kb
1.18kb
370bp

Fig. 5